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The use of symbiotic biodiversity to enhance plant tolerance to environmental stresses

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C) Executive Summary

In Uzbekistan, as in many other arid areas of the world, desertification is a process related to salt and drought plant stresses and is associated with considerable depletion of plant flora and soil microflora. According to international standards, Israel and Uzbekistan are considered arid and semiarid ecological regions, and both countries have similar need to arrest and restrain desertification, as well as to reclaim areas lost through desertification.

Acute problems of land salinization have been caused by inadequate irrigation in Uzbekistan, due to the very high evaporation coefficients and the low annual precipitation rates. The problem is aggravated by strong winds which carry salt dust from the dried up bed of the Aral Sea.

The importance of rizhosphere (plant root zone) organisms for the survival and productivity of plants in desert environments increases with the harshness of the environment. The relevance of these soil microorganisms for plants growing under all conditions is becoming increasingly evident, and they may be of critical importance in environments where the availability of water and/or mineral nutrient is limited. The desert areas of Uzbekistan are a reservoir of soil microorganisms capable of symbiotic associations with plant roots, even under saline and drought conditions. Some of the symbiosis mechanisms in desert conditions are poorly known, most are still unknown. The isolation, characterization, and inoculation of native symbiotic organisms to test plants would be very useful in current areas of interest, such as: arresting desertification, desert reclamation, crop production in arid areas, environmentally safe agriculture (lower use of fertilizers and pesticides), and ecosystem preservation. The purpose of this project was to establish the first necessary steps of selection, isolation, classification and characterization of native soil organisms, which stimulate plant growth and stress tolerance.

A collection of strains of nitrogen-fixing bacteria was isolated from leguminous plants of the Kyzil-Kum desert and, after detailed physiological and molecular studies, a number of strains was selected for further investigation of their effects on increasing productivity and plant tolerance to salt. Greenhouse and field experiments were carried out with native desert plants and with crop plants (wheat and alfalfa), at various salinity levels and irrigation regimes. The effects of commercially available inoculants were also studied. Yield increases of up to 38 % were obtained. In the laboratory, studies were conducted on some of the enzymes associated with nitrogen assimilation and stress mechanisms; the results suggest that the tested microorganisms contributed to the high stress tolerance of the plants.

The project has brought considerable benefits to the Uzbek partners. They have learned the advanced molecular biology techniques used to characterize the bacterial isolates. Their working visits to the Israeli laboratories allowed them to: (1) acquire hands-on experience on the use and care of modern laboratory instrumentation for molecular biology research, (2) use modern computers, including literature searches through electronic libraries and the Internet, and (3) further interact with the BGU team and prepare new research proposals (CA21-022, in pre-negotiation stage; CA23-042, decision pending). This project was their first attempt to plan and carry out a long-range research program in cooperation with a foreign team. Prior to the project, the Uzbek institution lacked mechanisms to administer project funds and a number of difficulties had to be overcome, some of them still persist. Thus, the equipment and the scientific and administrative skills acquired during this project have been an excellent foundation for future R&D work at the Uzbek institution, and are reflected on the increasing rate of success on mobilizing research funds from a number of sources.

D) Research Objectives

Uzbekistan is a relatively large country with considerable land resources, although much of its land is affected by salinity and drought. The biodiversity of its natural flora and soil microorganisms may hold a pool of genes expressing factors which enhance physiological and metabolic mechanisms of adaptation, factors that could eventually be transferred to ecosystems and crop genotypes to enhance their stress tolerance in marginal areas. This strategy would encourage farmers to remain in their increasingly infertile land and contribute to arrest the rapid desertification process of vast tracks of Uzbekistan, as well as of many other countries around the world. The project was a modest beginning, aiming at:

(a) Screening, classifying, and developing cultures and propagation methodologies for salt- and drought-resistant soil symbiotic microorganisms.

(b) Studying the increased stress-tolerance mechanisms these microorganisms can evoke in the plants with which they interact.

c) Increasing crop production and adaptation of shrubs and trees to ecosystems in areas affected by salinity, drought and low soil pH, through the association of stress tolerant soil microorganisms with plant roots.

E) Methods and Results

Materials and Methods

Plants: The plants studied were *Ammodendron conollyi* (sandy acacia), *Astragalus unifoliolatus* and *Astragalus villosissimus* (all shrubby leguminous plants of the Kyzil-Kum desert), in addition to the crops wheat and alfalfa.

Germination of plant seeds: The seeds from the indigenous leguminous plants showed a deep dormancy that had to be broken on the basis of preliminary tests using different treatments such as boiling, scarification, concentrated sulfuric acid, cutting of seed coat, use of hormones, seed priming with nitrate, etc. Seeds were scarified with concentrated H_2SO_4 (20 minutes for A. *villosissimus* and A. *unifoliatus*, and 40 minutes for A. *conollyi*), rinsed thoroughly with sterile distilled water and imbibed overnight on 1% (w/v) agar plates, followed by removal of the coat around the embryo. The seeds where then placed, embryo-side down, on agar plates and incubated at 30 °C for 2-3 days.

Isolation of nodule bacteria: The most appropriate time for nodule isolation was determined in a preliminary field study on the morphology of roots and nodules. Fresh nodules were detached in spring from newly formed lateral roots of the following Kyzil-Kum desert plants: *A. conollyi*, *A. unifoliolatus* and *A. villosissimus*. The detached nodules were washed repeatedly with sterile distilled water, sterilized with 96 % (v/v) alcohol, homogenized, and transferred onto sterile universal media for fast-growing and for slow-growing nodule bacteria. Colonies of fast-growing bacteria appeared after 2-4 days of incubation at 28 °C; colonies of slow-growing bacteria appeared up to 7 days of incubation. Purification of each bacterial isolate was repeated three times in the same medium. The purified strains were selected on the basis on the morphological characteristics of their colonies: sliminess, color, profile and transparency. A total of 219 isolates was obtained.

Detection of *nif* and *nod* genes and selection of strains: Because of the very high number of isolates to test, a modification of the colony-hybridization technique called patch-hybridization was used. Patches of isolates were made on solid medium in large (15 cm diameter) Petri dishes and

allowed to grow at 30 °C. When biomass was evident, a nylon membrane was cut to fit into the Petri dish and placed on top of the grown patches. The membrane with the biomass attached to it was treated for cell lyses and DNA fixation to the membrane, followed by hybridization to a labeled *nifHDK* probe (genes obtained from *Klebsiella pneumoniae*) and to a *nodABC* probe (genes originated from *Rhizobium meliloti*) by standard techniques (Maniatis *et al.*, 1982). *Klebsiella pneumoniae*, *Rhizobium meliloti*, and *Azorhizobium caulinodans* were used as positive controls, and *Escherichia coli* was used as negative control. The 219 isolates were analyzed by this procedure. Using the *nif* probe, 88 isolates produced a positive signal in the screening, while only 71 were considered as positive in the screening using the *nod* probe.
Finally, after testing for salinity resistance the following isolates were chosen for further studies: C8, C11, C15, and C21 obtained from nodules of *Ammodendron conollyi*; U7, U17, U30-1 and U30-2 isolated from nodules of *Astragalus unifoliolatus*, and V1, V3, V6, V8, V9, V9-1 and V30 from *Astragalus villosissimus*. Isolates were maintained in tryptone-yeast extract (TY) medium (Maniatis *et al.*, 1982).

Plasmid profile of the selected strains: The cellular plasmid contents of the isolates were visualized in 0.5% agarose gels by a modified Eckhardt technique. Plasmids of *Rhizobium leguminosarum* T3 were used as molecular size standards.

Phenotypic characterization of the selected bacterial strains: Growth rate at different concentrations of NaCl [0.04 (control), 0.75, 1.0, 1.5, 1.75 and 2.0 M], and at different pH values (4, 5, 6, 7, 8, 9, 10 and 11) were determined in TY liquid medium, at 30 °C. Growth at the temperatures of 12, 15, 20, 25, 30, 35, 37, 40 and 45 °C was determined in liquid TY medium, and growth at 4 °C was determined in TY solid medium. Resistance to antibiotics was tested by plating serial dilutions of the strains on TY agar medium containing ampicillin (10 μg/ml), chloramphenicol (20 μg/ml), kanamycin (30 μg/ml), streptomycin (50 μg/ml), or tetracycline (10 μg/ml). Melanin production was detected by the production of a diffusible dark pigment as described by Cubo *et al.* (1988). Bacteria were grown on TY plates supplemented with L-tyrosine (600 μg/ml) and $CuSO_4$ (40 μg/ml); fully-grown colonies were treated with 10% (w/v) sodium dodecyl sulfate (SDS) in TBE (Maniatis *et al.*, 1982) at pH 8.3. Strains failing to produce melanin were tested at higher concentrations of L-tyrosine (1.5 mg/ml) and $CuSO_4$ (80 μg/ml) (Cubo *et al.*, 1988).

Assay of ARA activity: Isolates were pre-grown in 5 ml of sterile semi-liquid medium, cell suspensions containing 10^8 cells ml^{-1} were prepared and 0.5 ml aliquots were transferred into 10 ml flasks. The flasks were hermetically closed with rubber caps and the air was removed with a syringe. After 4 days of growth at 30^0 C, acetylene (C_2H_2) was injected into the flasks to a final concentration of 10%. After 24 hours incubation, ARA (acetylene reductase activity) rates were determined using gas chromatography.

Glutamine synthetase: DEAE – Toypearl 650 M ion-exchanging chromatography was used to confirm the presence of multiple forms of glutamine synthetases in plant shoots. Enzymes elution was conducted with a linear gradient of NaCl (0.0 - 0.3 M NaCl).

Activities of aldehyde oxidase (AO) and xantine dehydrogenase (XDH). Enzyme extracts were obtained (from shoot, root and nodules) and assayed as described by Barabas *et al.* (2000) and Zdunek, and Lips (2001).

Effect of salinity on plant development and nodulation: Single sterile seedlings were planted in 250 ml pots filled with autoclave-sterilized dune sand, and the pots were irrigated with 20%

Hoagland medium (Hoagland and Arnon, 1938) containing 3 mM NH_4NO_3. From then on, NaCl was added to the nutrient solution in one of the following concentrations: 0 (control), 50, 100, 200, 300 and 500 mM. There were 10 replicate pots for each treatment. Irrigation prevented sand drying between nutrient applications, and the volume of fresh solution applied was above the field capacity of the pot substrate, thus preventing the accumulation of salts. After four weeks of treatment, plants were harvested, washed with distilled water, blotted on paper towel, separated into roots and shoots, and the length and fresh weight (FW) of roots and shoots were determined. The plant material was then placed in an oven for 48 h at 70 °C, allowed to cool to room temperature, and the dry weight (DW) was determined. Results are average values and for each treatment the standard deviations and levels of significance were analyzed by the Student T test using the program Microsoft Excel 2001.

Effect of drought on plant development and nodulation: Plants were pre-grown for 40 days in 6 l pots (6 plants per pot) filled with local soil and irrigated every two days with 10% complete Hoagland solution containing NH_4NO_3 in one of the following concentrations: 0 mM (control), 0.25 mM, 1 mM and 3 mM. The plants were then grown for further 32 days during which the interval between irrigations was 2 days (control), 4 days, 8 days or 16 days. Irrigation consisted of adding, all at once, 500 ml of nutrient solution per pot. Each treatment included six replicate pots. Harvest and processing of plant material was as described above.

Wheat Experiments: Preliminary laboratory and field studies were carried out in Uzbekistan on the effect of inoculating wheat with *Azorhizobium caulinodans* and *Glomus mosseae*. In order to simplify the presentation, all materials and methods used in these experiments are presented under this heading.

Azorhizobium caulinodans and *Glomus mosseae* were kindly donated by Prof. E. C. Cocking, (Department of Life Science, Center for Crop Nitrogen Fixation, Plant Genetic Manipulation Group, Nottingham University, Great Britain). *Azorhizobium caulinodans* was grown on solid TGYE medium, for 2-3 days at 28° C. Bacterial suspensions were then prepared with a density of 10^9 cells ml^{-1}, and an inoculum of 1 ml per plant was used. Inoculation with *Glomus mosseae* was achieved by placing 1 g of mycorrhiza preparation at a depth of 2-3 cm below the surface of the planting pots.

In the pot experiments, seeds of the soft variety "Dobraya" were sterilized by immersion in a 30 % solution of hydrogen peroxide, for 30 min; the process was repeated 6-10 times, followed by rinsing in sterile distilled water. Seed germination was carried out in Petri dishes, on sterile and humid disks of filter paper. Seedlings were planted at a depth of 0.5 cm in soil-containing pots, followed by inoculation. The soil was gray soil from a depth of more than 2 m, which made it poor in mineral nutrients. Treatments were as follows: nutrient solution without nitrogen (-N), nutrient solution with 70 ppm KNO_3 (+N), inoculation with *A. caulinodans* (-N+A), inoculation with *G. mosseae* (-N+M) and inoculation with both *A. caulinodans* and *G. mosseae* (-N+A+M). Each treatment comprised 10 pots with one plant each. Experiments lasted 70 days (from sowing till harvest of matured ears) under greenhouse conditions.

For the field experiments, seeds were mixed with the inocula prior to sowing in triplicate plots, and treatments were as in the pot experiments. Four wheat varieties were tested: a) soft wheat "Dobraya" and "Unumdor bugdoy" varieties, b) hard wheat "Carlic-85", and c) Triticosecale, a polyploid hybrid of wheat (Triticum) and rye (Secale). All wheat seeds were obtained from the Institute for Plant Breeding (Uzbekistan).

Alfalfa experiments: The alfalfa varieties "Tashkent-1728" and "Khorezm-2" were used in the study on the effects of salinity on seed germination and plant growth. Seeds were treated with concentrated sulphuric acid (Simarov, 1984), repeatedly rinsed with sterile distilled water,

transferred onto filter paper disks in Petri dishes, followed by the addition of 15 ml of a water solution containing 10 to 200 mM NaCl. After 15 min, the surplus of the solution was removed and the seeds were left on the impregnated filter paper. The Petri dishes were then placed in an incubator at 30 °C, and measurements of seed germination and root length were carried out every 24 hours.

The alfalfa variety "Khorezm-2" was used in sterile microvegetation experiments on the influence of salinity type (chloride and sulphate) on biomass production. Seeds were treated and germinated as above, 2-3 day-old seedlings were introduced into 60 ml agronomical tubes containing vermiculite impregnated with Hoagland solution, followed by inoculation with one or a combination of: a commercial preparation of *Glomus mosseae* (mycorrhiza), *Rhizobium meliloti* strain No. 24 (resistant up to 600 mM NaCl), and *Azospirillum* sp. A1-3 (resistant to 800 mM NaCl). The nutrient solution contained concentrations of NaCl varying from 30 to 100 mM, or Na_2SO_4 in concentrations from 5 to 50 mM. Plants were grown for 40 days in a greenhouse, under 14 hours daylight and at the temperature of 28 °C.

Results and Discussion

During the first year of the project, a large number of isolates were obtained from nodules of *Ammodendron conollyi, Astragalus villosissimus* and *Astragalus unifoliolatus,* all leguminous plants from the Kyzil-Kum desert, Uzbekistan. In spite of the sterilization of nodule surfaces, it is common to obtain rhizosphere bacteria which do not fix nitrogen. Thus, in order to select the true nitrogen fixers, a molecular approach was followed, based on the detection of nitrogen fixation genes (required for nitrogen fixation) and nodulation genes (required for symbiotic association). Detection of these genes was performed by hybridization ("patch hybridization") of total DNA from the bacterial strains to two probes, a *nifHDK* probe and a *nodABC* probe. Isolates that hybridized with both probes were then screened for salt tolerance as detected from their capacity to grow on TY solid medium containing up to 2 M NaCl. After this preliminary characterization, the 15 bacterial isolates listed in Table 1 were selected for further studies. Since symbiotic nitrogen-fixing bacteria bear megaplasmids required for symbiotic association, the plasmid content of the selected stains was examined. Plasmid electrophoresis showed that the isolates fell into two different profiles: strains C8-1, V1, V3, V8-1, V9 and V9-1 carried three plasmids of approximately 515, 370 and 118 kbp in size, respectively, while the smaller-size plasmid was absent in the remaining strains (Table 1).

Inspection of the isolates on TY medium showed at least 6 different types of colonies described in Table 1. All strains fell in the category of "fast growers" with doubling times ranging between 43 (C15) and approximately 78 min (C11). Three strains showed doubling times longer than 1 hour C11 (78 min), U30-2 (65 min) and V8-1 (76 min). Strain V6-1 had a doubling time of approximately 56 min and all remaining strains had doubling times of 50 min or less. In the presence of 0.75 M NaCl, doubling times were 1.4 to 1.9 longer than in control medium, and 1.6 to 4 times longer than in the control in the presence of 1.0 M NaCl. At 1.5 M NaCl differences in the tolerance to salt of the different isolates became more marked: V9-1 and V30 failed to grow, the doubling times of V3 an V8-1 were more than 26 times longer than those in control medium, while V9 was the isolate less susceptible to salt with a doubling time only two-times longer than that in the control medium. In the presence of 1.75 M NaCl strains U7 and V3 failed to grow, and a further increase on the doubling times of all remaining strains was observed. Finally, only strains C11, C15 and V9 grew at 2 M NaCl, with V9 showing a doubling time less than three times longer than that in the control medium; growth of strain C21 was extremely slow.

All isolates could grow in TY liquid medium at temperatures ranging from 12 to 40 °C, and V9 grew also at 45 °C. Except for V9, all isolates grew on agar plates at 8 °C. When cultivated in TY

Table 1: Selected isolates and some of their relevant characteristics.

Isolate	t_d[a] (h)	NaCl limit[b] (M)	Plasmid profile (kbp)[c]	Antibiotic resistance[d]	Growth temp. (°C)[e]	Growth pH[f]	Melanin production[g]	Colony type[h]
C8-1	0.82	1.75	370, 515	Amp	10 - 40	4 – 11	-	I
C11	1.31	2.00	370, 515	Cm	10 - 40	6 – 10	weak	II
*C15	0.72	2.00	118, 370, 515	Amp	10 - 40	4 – 11	-	I
C21	0.68	1.75	370, 515	Amp	10 - 40	4 – 11	-	I
*U7	0.84	1.5	370, 515	Amp, Km, Str, Tc	10 - 40	4 – 11	+	I
U17-1	0.84	1.75	370, 515	Amp, Tc	10 - 40	4 – 11	+	I
*U30-1	0.77	1.75	370, 515	Amp, Km, Str, Tc	10 - 40	4 – 11	+++	III
*U30-2	1.08	1.75	370, 515	Amp, Tc	10 - 40	4 – 10	+	IV
V1	0.81	1.75	118, 370, 515	Amp, Tc	10 - 40	4 – 11	+	I
V3	0.76	1.50	118, 370, 515		10 - 40	4 – 11	-	II
V6-1	0.93	1.75	370, 515	Amp, Tc	10 - 40	4 – 11	+	I
V8-1	1.27	1.75	118, 370, 515	Amp, Cm	10 - 40	5 – 11	-	I
V9	0.75	2.00	118, 370, 515		12 - 45	4 – 11	-	V
*V9-1	0.84	1.0	118, 370, 515		10 - 40	5 – 11	-	IV
V30	0.81	1.0	370, 515	Amp, Cm, Km, Str	10 - 40	4 – 11	++	VI

[a] Doubling time in TY liquid medium at 30 °C.

[b] Highest concentration of NaCl in TY liquid medium at which growth was observed. The following were tested at 30 °C: 0.04 (control), 0.75, 1.0, 1.5, 1.75 and 2.0 M NaCl.

[c] Plasmid patterns were detected in Eckhardt gels (0.5% agarose) and the sizes of the plasmids were estimated from their migration distances and the plasmids of *R. leguminosarum* T3 as molecular size standards.

[d] The following were tested in TY solid medium: ampicillin (10 µg/ml), chloramphenicol (20 µg/ml), kanamycin (30 µg/ml), streptomycin (50 µg/ml) and tetracycline (10 µg/ml).

[e] Growth at 10 °C was tested in TY agar plates, and growth at 12, 15, 20, 25, 30, 35, 37, 40 and 45 °C was tested in TY liquid medium

[f] Growth was tested in TY liquid medium at the following at the pH values: 4, 5, 6, 7, 8, 9, 10 and 11.

[g] Detected by a diffusible dark pigment when grown in TY plates supplemented with L-tyrosine and $CuSO_4$ (Cubo *et al.*, 1988).

[h] Type I: Medium-size, round, convex, shiny and white.
Type II: Young colonies as I, becoming yellow and umbonate later.
Type III: Young colonies as I, becoming beige later.
Type IV: As type I but smaller.
Type V: Medium, round, white, dull and curled.
Type VI: Young colonies as I, later becoming darker than III.

liquid media adjusted to pH increments of one unit in the range of 4 to 11, most isolates could multiply. Exceptions were isolates V8-1 and V9-1, which failed to grow at pH 4, C11 which grew only from pH 6, and U-30-2 which only grew until pH 10 (Table 1). Growth in Luris-Bertani medium was observed for all isolates.

Strains of rhizobia differ in their natural resistance to various antibiotics, a characteristic which may provide a means of identifying closely related strains. Preliminary screening of antibiotic resistance showed that although colony numbers may have decreased somewhat in the presence of chloramphenicol (and occasionally with other antibiotics) the resistance patterns presented on Table 1 existed. Capacity to grow on plates supplemented with ampicillin was the most widely distributed, followed by that to grow in the presence of tetracycline. Isolates U7, U30-1 and V-30 grew in the presence of four of the antibiotics. Another phenotypic characteristic that can assist on the identification of closely related rhizobia is the production of melanin. Rapid and abundant production was observed by isolate U30-1, closely followed by isolate V30; less abundant production was detected for isolates U7, U17, U30-2, V1 and V6. The remaining isolates failed to produce a diffusible dark pigment and were considered to be Mel$^-$ (Table 1).

Table 2: Acetylene-reductase activity of nodule bacteria grown in a nitrogen-free liquid medium. Strains were isolated from *Ammodendron conollyi*, *Astragalus villosissimus* and *Astragalus unifoliolatus* and *Azospirillum brasilense* sp 7. ATCC 29145 was used as control.

Bacteria	**Acetylene reduction activity nmol C_2H_4 flask^{-1} hour^{-1}**
V1	0.39
V3	0.31
V6[1]	trace
V8[1]	0.52
V9	0.47
• V9[1]	0.39
V30	trace
C8[1]	0.34
C11	0.21
• C15	trace
C21	0.18
• U7	trace
U17[1]	0.34
• U30[1]	0.16
-U30[2]	trace
Control	140.40

Although the nodule bacterium *Azorhizobium caulinodans* appears to be able to fix molecular nitrogen both in the free-living state and in symbiotic association, no evidence is available on the ability of other nodule bacteria to fix molecular nitrogen out of their symbiotic state. Our selected bacterial strains grown in nitrogen-free nutrient medium (except for N_2) were tested for N_2-fixing activity using the free-living nitrogen-fixing organism *Azospirillum brasilense* sp 7. ATCC 29145 as reference (Table 2).

When detected, acetylene loss was too low compared to that of *Azospirillum brasilense* sp 7. ATCC 29145 and may be attributed to experimental variability. In their free state, the bacteria tested appeared to be unable to fix N_2.

Seeds of *Ammodendron conollyi*, *Astragalus villosissimus* and *Astragalus unifoliatus* showed a deep dormancy which was broken as described above. In these plants, root development exceeded by far that of the shoots (Figs 1-3). Among the three plant species, *A. conollyi* showed the lowest salt resistance, and significantly reduced growth in the presence of 50 mM NaCl, with no surviving plants in the presence of 100 mM NaCl or above (Fig 3). When compared to the *Astragalus* plants, *Ammodendron conollyi* plants were considerably more sensitive to 50 mM NaCl in all growth parameters measured: root and shoot fresh and dry mass, as well as root and shoot length. The potential biomass production capacity of these plants would require optimization of nutritional and irrigation management, as well as selection of specimens with higher dry-mass productivity.

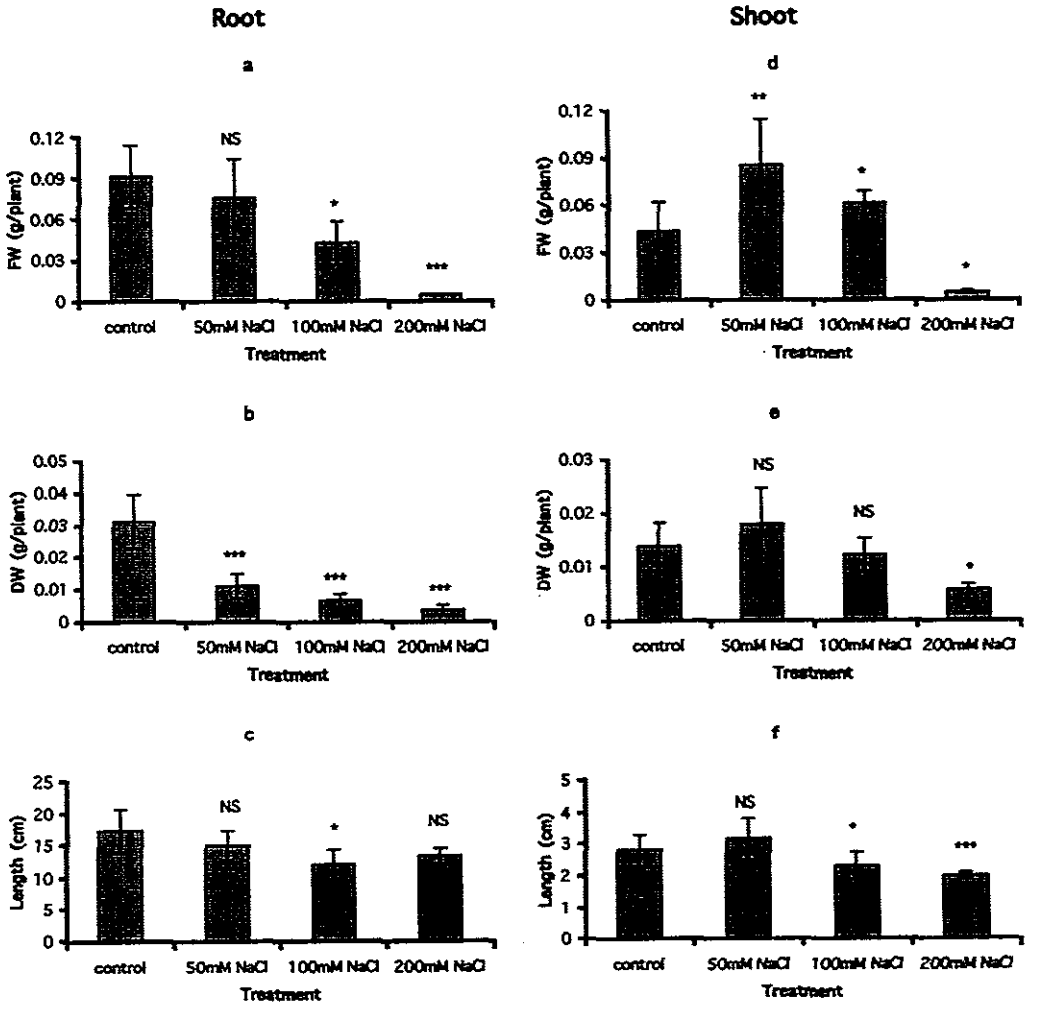

Fig. 1: Effect of salinity on the development of five-week old *Astragalus unifoliatus* plants.

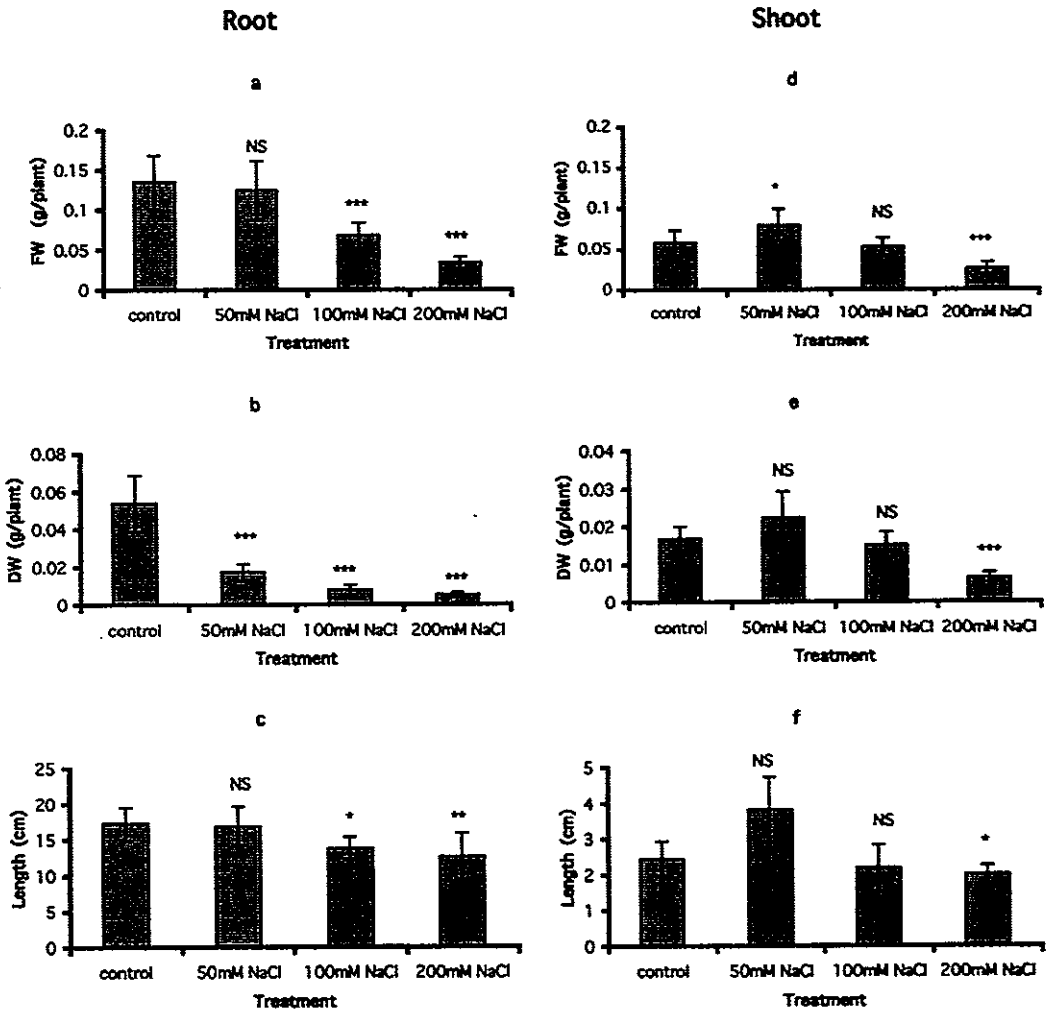

Fig. 2: Effect of salinity on the development of five-week old *Astragalus villosissimus* plants.

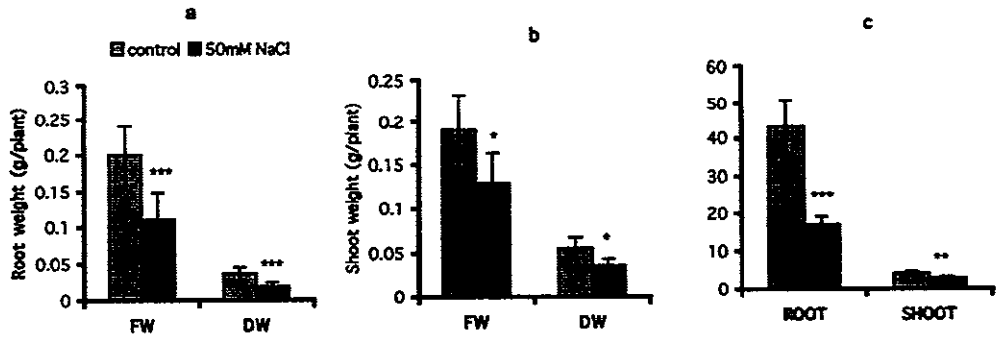

Fig 3: Effect of salinity on the development of five-week old *Ammodendron conollyi* plants.

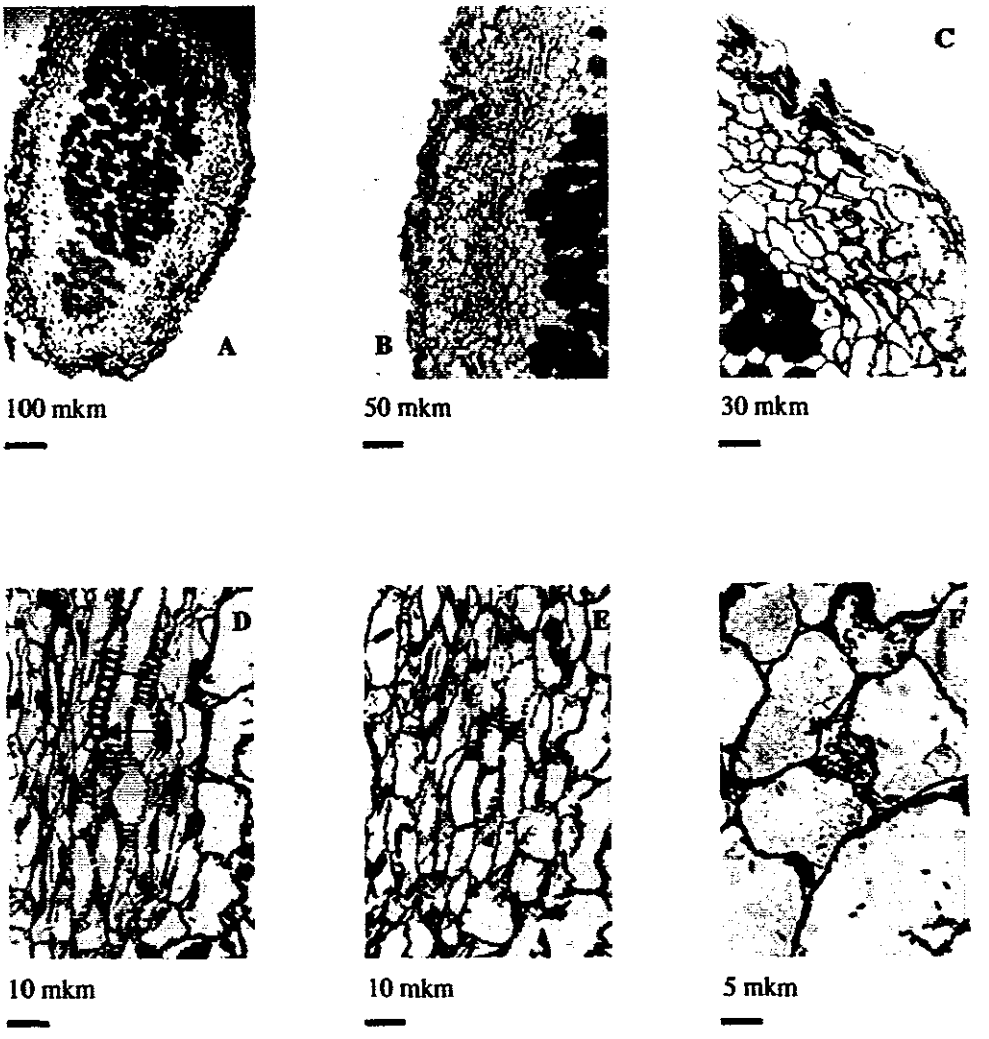

Fig. 4: Light microscopy of nodule sections of an *Astragalus villosissimus* plant inoculated with strain V1, isolated from the same plant species:
A - Longitudinal section of nodule (x148 magnification).
B - Higher magnification of section (x315). Visible central nodule plant cells, densely colonized by bacterial cells, and also outer plant cortex nodule cells and the zone of formation and development of infection thread centers.
C - Magnification (x530) of the same section showing outer bacterial net in the plant cover.
D and E - Higher magnification (x1974) of the infection thread showing "stair-like" intracellular infection threads (pointed out by arrow) and some elements of intercellular infection threads (globular dark red structures).
F - The highest magnification (x2835); visible development and expansion of thick infection threads along plant nodule membranes and across nodule cells together with released cells of nodule bacteria.

In general, the effects of salinity on the development of *A. unifoliatus* (Fig. 1) and *A. villosissimus* (Fig. 2) were similar and no plants survived at 300 and 500 mM NaCl. The dry and fresh weights of the shoots of plants grown in the presence of 50 and 100 mM NaCl were higher or not significantly different from those of the control plants (Figs 1 and 2). It was interesting to observe that even when grown in the presence of 200 mM NaCl and with a drastic loss of dry matter production, plant length was affected to a much lower extent; this was possible by a more focused allocation of dry matter to the central shoot and by cutting down on side growth. This effect was evident both on root and shoot development. Also notable is the increase in dry matter production of the shoot of plants kept under 50 mM NaCl, indicating a reverse of the traditional trend shown by stressed plants in which shoot growth is inhibited more than root growth. All these changes indicate that these plants have the capacity to change the patterns of dry mass distribution under increasing saline conditions in order to survive and produce seeds.

Experiments were then set up with the objective of testing the effect of inoculation and nodulation on the development of the three plant species. During the first week, the seedlings received 3 mM NH_4NO_3, and then they were inoculated with one of the 15 bacterial strains by dispensing, close to the root, 2 ml of a suspension with approximately 10^9 cells ml^{-1}. From the time of inoculation, the concentration of nitrogen in the nutrient solution was lowered to 1 mM NH_4NO_3. Two weeks later, NaCl was added at the concentrations of 50, 100 and 300 mM for *A. unifoliatus*, of 100 and 300 mM for *A. villosissimus*, and of 50 and 100 mM for *A. conollyi*. For each plant-bacterium combination there were controls with or without nitrogen in the nutrient solution, and controls without added NaCl. Un-inoculated controls were also set up for each plant species. The highest level of NaCl tested was fatal. Plant development at all other concentrations was similar to that shown in Figs 1-3. Root nodules were not found in any of the plants, and the experiments were repeated twice with the identical results. However, nodulation was observed in other sets of experiments. These results were detailed in the first yearly report and a representative illustration is shown in Fig. 4.

Table 3: Glutamine synthetase activity of *Ammodendron conollyi, Astragalus villosissimus* and *Astragalus unifoliolatus.*

Plant	*Specific activity mmol product mg^{-1} protein min^{-1}		
	Control	100 mM NaCl	% inhibition
Ammodendron conollyi, shoot	0.82	0.69	16
root	0.24	0.19	21
Astragalus villosissimus, shoot	1.30	0.96	26
root	0.30	0.25	17
Astragalus unifoliolatus, shoot	1.47	1.09	26
root	0.40	0.30	25

*GS product was g-glutamilhydraxamic acid formed in the course of the reaction.

Glutamine synthetase (GS) is a key-enzyme on the assimilation of ammonium and a further synthesis of nitrogenous metabolites. GS is subject to strict regulation both at the transcription and translation level as well as to post-translational regulation. Ammonium plays an important role in the regulation of the synthesis and activity of GS. Ammonium in plant roots is a product of nitrogenase reduction of atmospheric N_2 or from direct uptake from the soil.

Plants grown in medium with 100 mM NaCl showed root and shoot GS activities 16% to 26% lower than that those in control (no salinity) plants (Table 3). For each plant species, the GS activity in the shoot was three times higher than in the root, both in the presence and in the absence of salinity.

Different isoforms of GS have been found in a large number of plant and bacterial species. For example, two forms of GS were described in pea and pumpkin leaves (GS_1 and GS_2), one of the isoforms was located in chloroplasts and the other in the cytosol; cells of *Bradyrhizobium* sp. in N_2-fixing nodules of lupine contained at least three GS isoforms, while cells from inefficient nodules had only two isoforms. GSs with similar catalytic activities frequently differ in their amino acid composition, molecular weight and a variety of other properties (specific activity, optimum pH, thermostability, etc.). These differences are linked to enzyme location (e.g., cytosol, chloroplast). Taking this into account, we decided to study the multiple molecular forms of GS both in the test plants and in the isolated bacterial strains.

Shoot extracts of *Ammodendron conollyi* and *Astragalus unifoliolatus* contained two isoforms of GS with different activities and electrophoretic mobility detected by PAGE. Only one form of GS was detected in the shoots of *Astragalus villosissimus* and in the roots of each of the species studied (Fig. 5). The isoform with smaller electrophoretic mobility was designated GS_1, and that with the greater mobility as GS_2. Practically all GSs detected in the zymograms of plant shoots had a high level of electrophoretic mobility and activity, while roots GS displayed low activity. The activity of GS_2 was several times higher than that of GS_1. The first peak eluted with 0.06 M NaCl and the second at 0.18 M NaCl. Shoots of *Astragalus villosissimus* had very low GS_1 activity, barely detectable in the PAGE zymograms

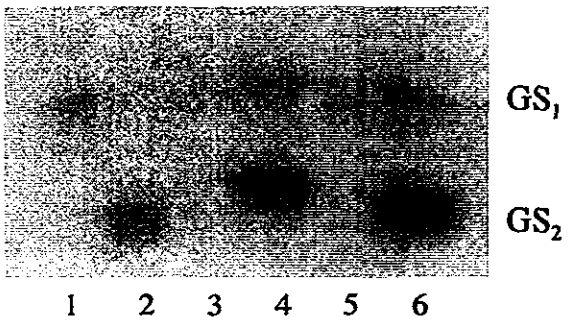

Fig. 5: Zymogram of GS in extracts of: (1) root and (2) shoot of *Astragalus villosissimus*, (3) root and (4) shoot of *Astragalus unifoliolatus*, and of (5) root and shoot (6) of *Ammodendron conollyi*.

A comparative study was carried of the physical and chemical properties of *Ammodendron conollyi* shoot GSs (GS_1 and GS_2) and of GS from shoot and root of *Astragalus villsiossimus*. Maximum activity of GS_1 was observed at pH 6.2, while maximum activity of GS_2 and of root and shoot GS of *Astragalus villsiossimus* was at pH 6.4 (Fig. 6a).

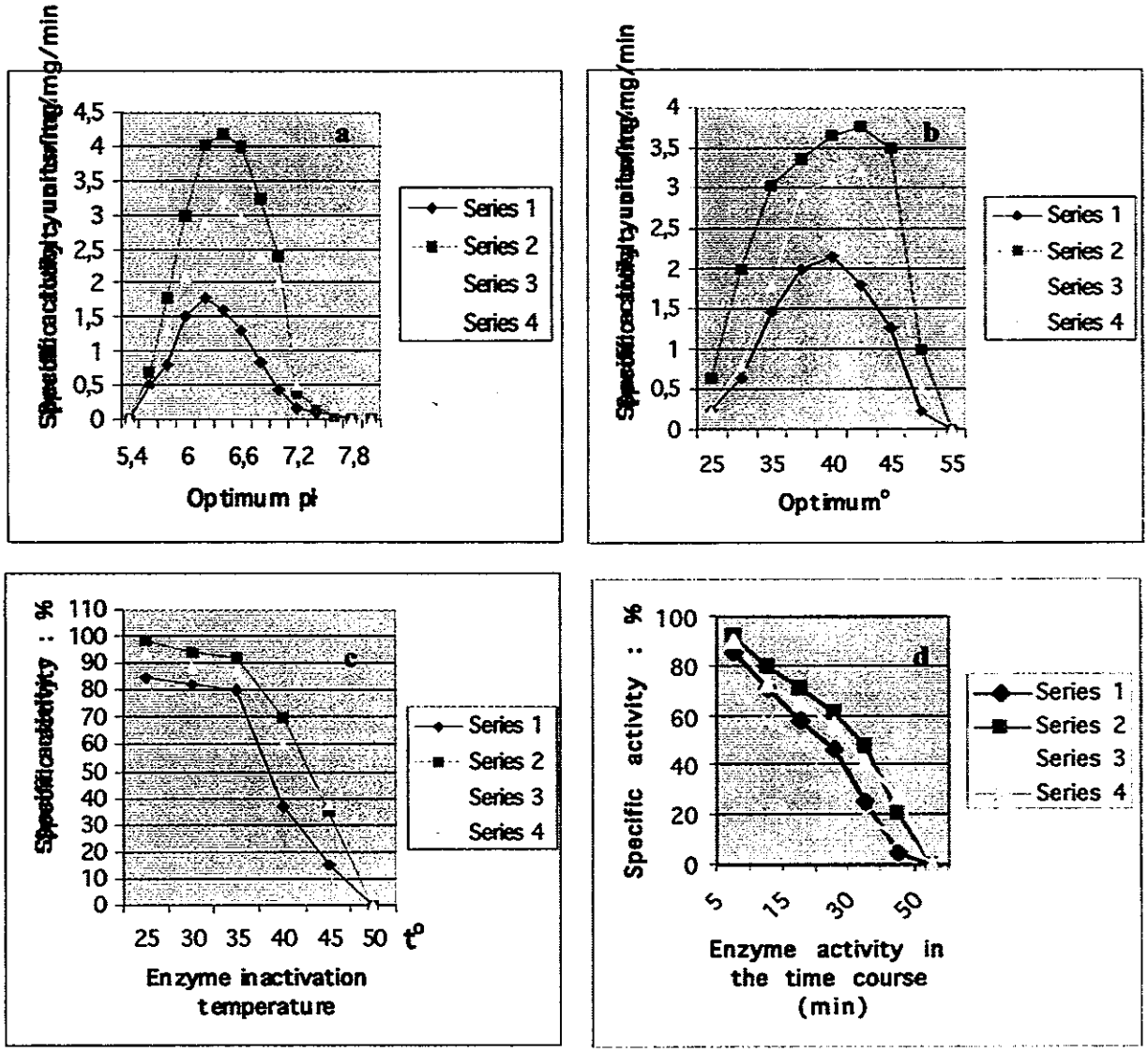

Fig. 6: Physical and chemical properties of plant glutamine synthetase:
Series 1 – GS_1 from *Ammodendron conollyi*
Series 2 – GS_2 from *Ammodendron conollyi*
Series 3 – GS from shoot of *Astragalus villosissimus*
Series 4 – GS from root of *Astragalus villsiossimus*
Specific activity: the amount of g-glutamilhydraxamic acid formed in the course of reaction (units/mg/min).

Table 4: Total activity of glutamine synthetase in nodule bacteria

Bacterial isolate	Specific activity: mmol GH mg^{-1} protein min^{-1}		
	+ N	- N	+ N + 1 M NaCl
V1	2.37	2.51	2.18
V3	2.43	2.71	2.26
$V6^1$	1.98	2.17	1.81
$V8^1$	2.70	2.81	2.50
V9	2.54	2.85	2.45
$V9^1$	2.59	2.93	2.37
V30	2.19	2.39	2.10
$C8^1$	2.21	2.39	2.00
C11	2.28	2.59	2.37
C15	1.87	2.09	1.90
C21	2.20	2.32	2.10
U7	2.14	2.27	2.07
$U17^1$	1.66	1.89	1.49
$U30^1$	1.60	1.73	1.51
$U30^2$	1.80	1.87	1.66
CXM1*	2.90	3.60	2.62

**Rhizobium meliloti* CXM1

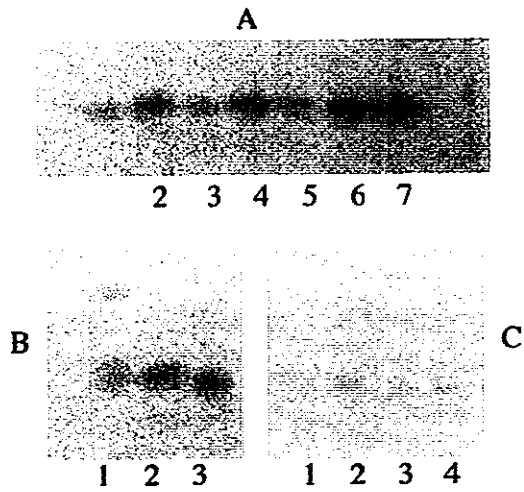

Fig. 7: Zymograms of GS in bacterial extracts:
A) Nodule bacteria from *Astragalus villosissimus*: 1 – V30; 2 – $V9^1$; 3 – V9; 4 – $V8^1$; 5 – $V6^1$; 6 – V3; 7 – V1
B) Nodule bacteria from *Astragalus unifoliolatus*: 1 – $U17^1$; 2 – U7; 3 – $U30^1$
C) Nodule bacteria from *Ammodendron conollyi:* 1 – C15; 2 – $C8^1$; 3 – C11; 4 – C21

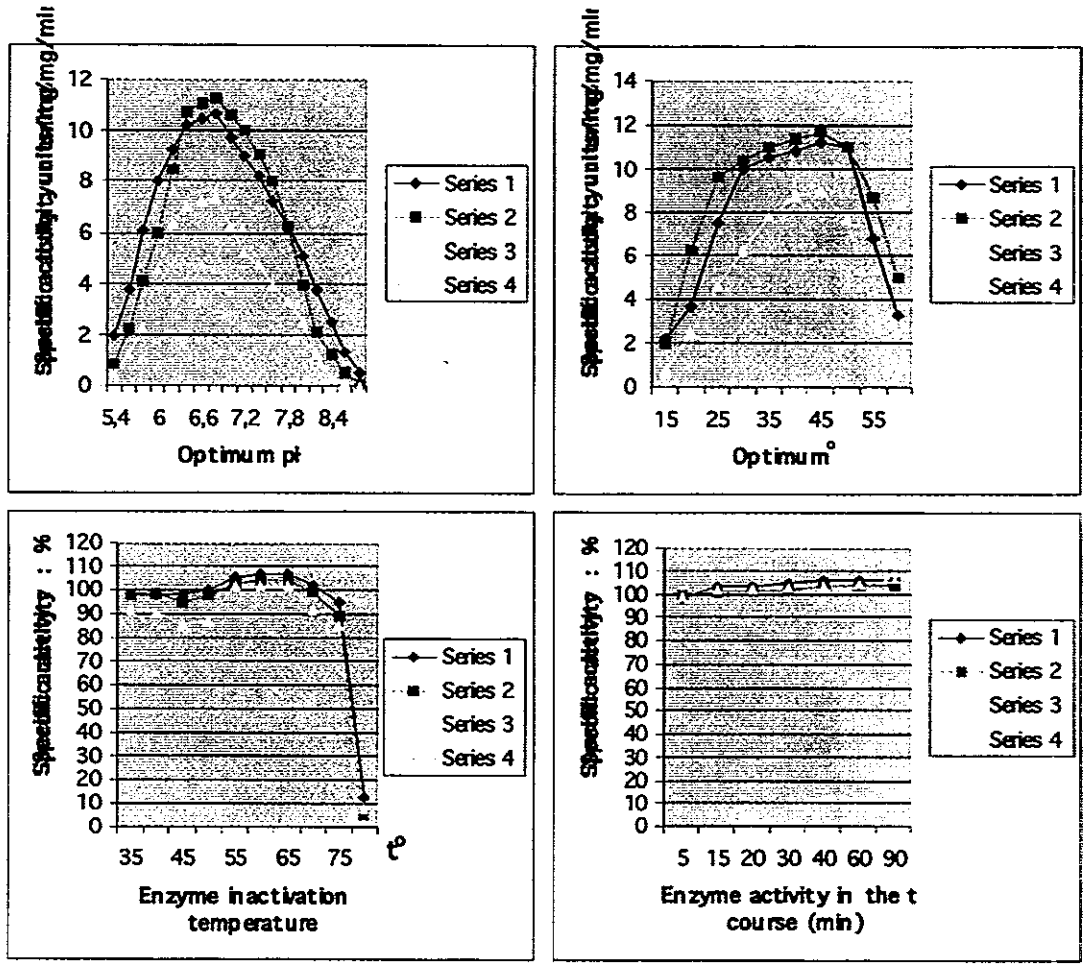

Fig. 8: Physical and chemical properties of nodule bacteria glutamine synthetase:
Series 1: V1 (isolated from *Astragalus villosissimus)*
Series 2: V3 (isolated from *Astragalus villosissimus)*
Series 3: C8[1] (isolated from *Ammodendron conollyi)*
Series 4: • U7 (isolated from *Astragalus unifoliolatus)*
Specific activity: the amount of g-glutamilhydraxamic acid formed in the course of reaction (units/mg/min).

The optimal assay temperature for all GS isoforms was 40 - 43 ^{0}C (Fig. 6b). *Ammodendron conollyi* GS_2 and *Astragalus villossimus* shoot GS lost approximately 30 - 40% of their activity after heating to 40 ^{0}C, while *Ammodendron conollyi* GS_1 and *Astragalus villsissimus* root GS lost up to 70% activity under the same conditions (Fig. 6c). Considerable inactivation was observed in all GSs heated at 37 ^{0}C and total inactivation occurred after 50 minutes at 50 ^{0}C (Fig. 6d).

Except for U17[1], which produced 3 GS isoforms, all bacterial strains cultivated for three days in a leguminous broth showed only one form of GS when analyzed by PAGE (Fig. 7). However, a single form of GS was found in all strains when grown in the presence of 1 M NaCl, and all GSs had the same electrophoretic mobility. A more detailed study using DEAE confirmed the results obtained with PAGE. Only one peak of GS appeared after DEAE. Proteins with GS activity eluted from the column at high concentration of NaCl (0.345 M NaCl). For example: maximum ionic power recorded for the elution of GS was 0.24 M NaCl. Strains U17[1] and CXM1 had three forms of GS.

The optimum pH for GS isolated from strains V1, V3, C8[1] was 6.8, and was 7.0 for that from U7[1] (Fig. 8a). The optimal temperature was in the range of 40 - 50 ^{0}C (Fig. 8b). Enzyme activity was either unchanged after heating for 1.5 h at 50 ^{0}C (Fig. 8d), or even increased in strains V1, V3 and V8. Further heating up to 65 ^{0}C did not cause enzyme inactivation, with GS of strains V1, V3 and C8[1] showing a slight (7%) increase in activity in relation to their initial activity (Fig. 8c).

Thus, GSs isolated from plants and nodule bacteria exhibited considerable differences in their physical and chemical properties, especially in their stability (Figs 6d and 8d) and inactivation temperature. While the activity of the plant GS declined rapidly in the assay medium, the activity of the bacterial GS was sustained throughout the period tested (50 min). Addition of 1M NaCl to the growth media for 24 hours prior to assay reduced the activity of GS by less than 8 %. **This observation suggests that nodule bacteria of desert plants have a high stress tolerance facilitating plant survival under very harsh conditions.**

One of the project tasks was to assess the effects of inoculation on the development of wheat and alfalfa, two important crops in the target country. The nodule bacterium *Azorhizobium caulinodans* and the mycorrhiza *Glomus mosseae* were tested, under greenhouse and field conditions. *Azorhizobium caulinodans* forms nitrogen-fixing nodules on roots and stems of the tropical legume *Sesbania rostrata*, and is also capable of symbiotic colonization of roots of non-legumes (wheat, rice, *Arabidopsis thaliana*, etc.) assimilating N_2 (Cough *et al.*, 1996). This nodule bacterium fixes N_2 not only in the bacteroid state within the host plant nodule but also in its free-living state (Mergaert *et al.*, 1995).

Mycorrhizae are symbiotic fungi that interact with higher plant roots. Endomycorrhiza are capable of establishing symbiotic interactions with 70 % of the vascular plants, penetrating and growing within the plant root cells. The expected benefits of mycorrhiza are: a) concentration and accumulation of phosphorous and other essential mineral nutrients in poor soils, b) protection against pathogenic soil fungi, c) cellulase and pectinase activities which may play a role in the opening and loosening of the root surface thus facilitating the formation of nitrogen-fixing nodules on roots of non-legumes. Wheat was found to be responsive to inoculation by *Azorhizobium caulinodans* (Cough *et al.*, 1996). At the same time, roots of wheat were colonized by arbuscular mycorrhiza to an extent of 40-76 % (Niemira *et al.*, 1996).

A preliminary experiment was carried out to test the effect of *Azorhizobium caulinodans* and *Glomus mosseae* (endomycorrhiza) on the growth and yield capacity of different wheat varieties. During the first week in greenhouse experiments, plants inoculated with *Azorhizobium caulinodans* grew slower than non-inoculated plants and plants with mycorrhiza only. By the third week, there were no differences in height between inoculated and non-inoculated plants. On the fifth week, +N non-inoculated plants and all inoculated plants were 1.5 times higher than -N plants. Development of ears began in the -N control 5-7 days later than in all other treatments. On the 59th day all plants attained the same height, and on the 70th day all ears were harvested.

Table 5: Effect of added nitrogen, *Azorhizobium caulinodans* (+A) and *Glomus mosseae* (+M) on the production of wheat biomass and grain (pot experiment).

Treatments	Root mg DW	Shoot mg DW	Grain mg DW	Biomass mg DW	Grain % of -N	Grain # ears
-N	236	179	97	512	100	3.2
+N	265	127	309	701	320	12.3
-N+A	265	138	313	716	324	12.2
-N+M	282	139	333	754	345	12.1
-N+A+M	297	143	355	795	367	13.8

The highest average biomass of dry shoot was in the treatments in which seeds received a combined inoculum of bacterium and mycorrhiza (Table 5). At the same time, the average dry mass of roots treated with mycorrhiza was about 19-25 % higher than in the other treatments.

Table 6: Effect of *Azorhizobium caulinodans* (+A) and *Glomus mosseae* (+M) on grain yield of three wheat varieties under field conditions (three replicate plots).

Wheat Variety	Treatments	Grain yield (g/m^2) 1	2	3	Average	% of control
Soft wheat "Unumdor Bugdoy"	-N (control)	683	630	719	677	100
	-N+A	768	817	793	792	117
	-N+M	779	785	712	758	112
	-N+A+M	865	809	827	834	123
Hard wheat "Carlic-85"	-N (control)	437	305	483	408	100
	-N+A	501	460	493	484	119
	-N+M	490	457	477	474	116
	-N+A+M	529	548	610	562	138
Triticosecale	-N (control)	764	552	627	614	100
	-N+A	800	681	729	736	120
	-N+M	611	825	764	666	109
	-N+A+M	771	885	780	812	133

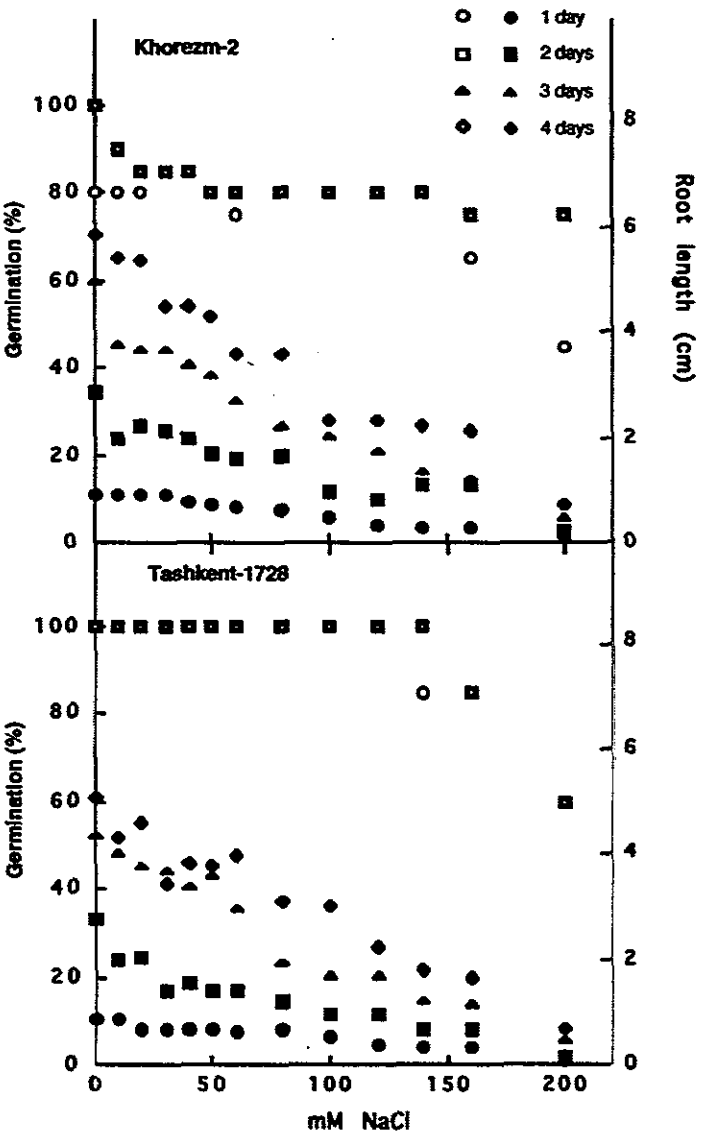

Fig. 9: Effect of salinity on the germination and development of alfalfa (var. Khozem-2 and Tashkent-1728). Open symbols, germination; closed symbols, root length.

Thus, mycorrhiza appeared to have promoted a more extensive development of wheat roots at the expense of shoot biomass. The yield capacity of wheat was estimated by two parameters: the average number of grains per ear, and the average mass of ears. **The results obtained indicated a remarkable enhancement of grain yield production by the addition of the N_2-fixing bacterium and the mycorrhiza under conditions of limiting nitrogen in the growth substrate of the plants.**

Table 7: Dry biomass of alfalfa plants under different salinity treatments in the presence and in the absence of medium nitrogen, and inoculated with *Glomus mosseae* (mycorrhiza), *Rhizobium meliloti* strain No. 24 and *Azospirillum* sp. A1-3. Control plants were not inoculated nor subjected to salt treatment. Results are average of 10 plants (5 pots with two plants each).

Treatment		Plant dry weight (mg)									
		No Salinity	mM Na_2SO_4					mM NaCl			
			5	10	20	30	50	10	30	50	80	100
- Nitrogen	Rhizobium Mycorrhiza	25.0 (130)*	26.3 (136)	22.3 (116)	17.4 (90)	13,0 (67)	13,8 (72)	25,5 (131)	24,3 (126)	19,7 (102)	17,1 (89)	13,4 (69)
	Rhizobium Mycorrhiza Azospirillum	26.6 (138)	21.9 (114)	21.3 (110)	17,7 (92)	11,6 (60)	9,3 (48)	24,2 (125)	21,8 (113)	20,9 (108)	16,0 (83)	11,3 (59)
	Rhizobium Azospirillum	23.7 (123)	22.0 (114)	22,5 (117)	15,9 (82)	13,1 (68)	12,9 (67)	24,1 (125)	23,6 (122)	23,0 (119)	16,1 (83)	13,0 (67)
	Rhizobium	22.2 (115)	19.4 (101)	19.2 (100)	17,6 (91)	12,0 (62)	11,2 (58)	23,2 (120)	20,3 (105)	16,3 (84)	15,8 (82)	12,1 (63)
	Control	19,3 (100)	18.5 (96)	18.3 (95)	14,2 (74)	11,4 (59)	10,5 (54)	20,1 (104)	19,0 (98)	17,0 (88)	13,1 (68)	9,5 (49)
+ Nitrogen	Rhizobium Mycorrhiza	35.6 (125)	23.5 (82)	21.0 (73)	17,5 (61)	12,2 (43)	11,7 (41)	30,7 (109)	25,3 (90)	20,1 (72)	15,3 (54)	10,0 (36)
	Rhizobium Mycorrhiza Azospirillum	36.0 (126)	(29) (101)	18.9 (70)	18,3 (64)	14,3 (50)	11,9 (42)	31,3 (109)	27,1 (95)	23,0 (80)	17,9 (63)	11,5 (40)
	Rhizobium Azospirillum	32.4 (113)	23.0 (80)	18.9 (66)	16,8 (59)	13,5 (47)	12,7 (44)	30,0 (105)	24,6 (86)	21,3 (74)	18,3 (64)	11,9 (42)
	Rhizobium	28.9 (101)	23.7 (83)	19.5 (68)	17,1 (60)	14,1 (49)	12,2 (43)	27,1 (95)	25,7 (90)	20,1 (70)	16,3 (57)	13,1 (46)
	Control	28,6 (100)	24.3 (85)	19 (66)	17,3 (61)	15,5 (54)	13,0 (46)	28,1 (98)	23,1 (81)	18,4 (64)	15,1 (53)	14,8 (52)

* Values between brackets are percentage of non-inoculated controls.

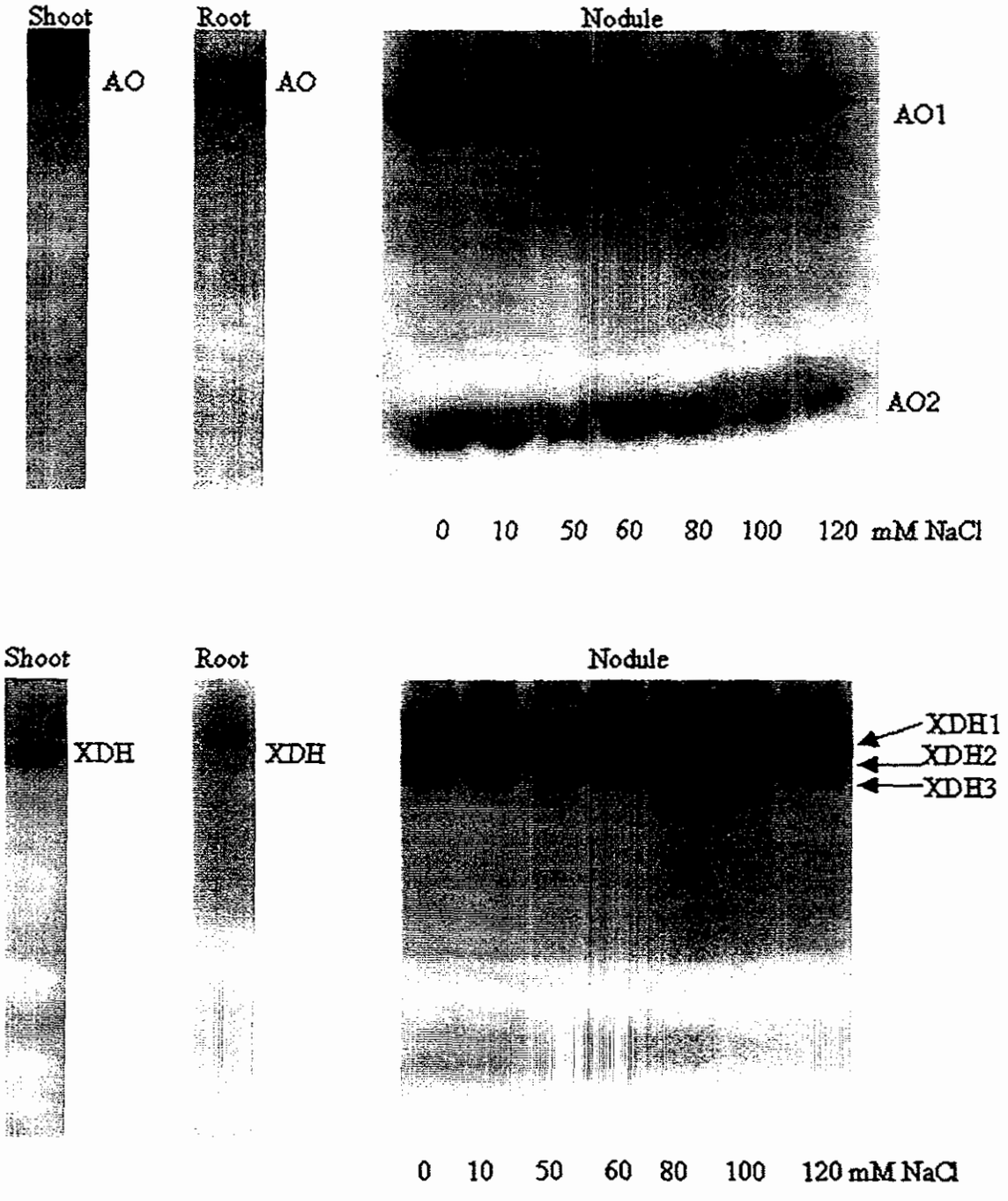

Fig. 10: AO and XDH activities in different parts of alfalfa (shoot, root and nodule) grown in different salinity range of NaCl. Supernatant samples (100 µl/ ane) were subjected to native PAGE, followed by AO- and XDH activity staining with acetaldehyde and hypoxanthine as substrate.

A similar experiment was conducted with 3 varieties of wheat under field conditions: soft wheat "Unumdor bugdoy", hard wheat Carlic-85 and Triticosecale (Table 6). Additions of *Azorhizobium* and mycorrhiza to wheat seeds caused an increase of grain yield of the varieties tested. The effects are lower than those observed in the pot experiments; this may be explained by higher control values due to possible higher level of N in the soil, or may be related to species differences.

The inoculation of wheat with nodule bacterium led to a yield increase of 17-20 % above the control in all varieties tested. The highest inoculation effect was with the combined treatment of bacterium and mycorrhiza, which brought about a yield increase of 23-38 % as compared to the controls.

On the basis of the results obtained under greenhouse and field conditions one can conclude and confirm that the nodule bacterium *Azorhizobium caulinodans* and the mycorrhiza *Glomus mosseae* promote growth and grain yield of wheat plants. The economic importance of these effects is presently under consideration.

The effect of salinity on the germination of alfalfa was also studied. Seeds of two alfalfa varieties were subjected to treatment with NaCl at concentration up to 200 mM, for 4 days. In general, the analysis of the percentage of seed germination showed that at the highest concentrations of NaCl Khorezm-2 was less affected by salt than "Tashkent-1728". Although the length of the roots gradually decreased with increased salinity, the effect was less pronounced in Khorezm-2 (Fig. 9). In Uzbekistan, "Khorezm-2" is considered to be a salt-resistant and arid variety.

Sulphate (Na_2SO_4) salinity is reportedly more harmful than chloride (NaCl) salinity, and its effects are noticeable at concentrations lower than those of chloride salinity (Jaiwal *et al.*, 1997); a comparative study was carried out on the effects of chloride and sulphate salinity on the early development of alfalfa seedlings, and the results obtained confirmed the pattern (Table 7).

AO (aldehyde oxidase, EC 1.2.3.1) and XDH (xantine dehydrogenase, EC 1.2.1.37) play an important role in the adaptation of plants to environmental stresses (Barabas *et al.*, 2000; Zdunek and Lips, 2001) and that changes of their activities and diversity of their molecular isoforms could reflect the adaptation capability of a plant to different environmental and physiological stresses. A study was undertaken of AO and XDH activities in different parts (shoot, root and nodules) of alfalfa plants grown a salt concentrations up to 120 mM NaCl (Fig 10).

One isoform with low XDH activity was detected in the shoot and root, whereas three XDH isoforms with similar electrophoresis mobility were observed in nodules: a high activity (XDH1) form and two lower activity (XDH2 and XDH3) forms, which had similar electrophoresis mobility, The activities of XDH2 and XDH3 increased a levels of salinity in the range of 80-120 mM. Root and shoot showed only one form of AO while two forms with very different electrophoretic mobility were detected in the nodules. **Changes of their activities and diversity of their molecular isoforms could be markers of plant adaptation capability to different environmental and physiological stresses**

F) Impact, Relevance and Technology Transfer

The Uzbek PIs have learned advanced molecular biology techniques through their active participation in the characterization of the bacterial strains isolated from the target leguminous plants. Their visits to the Israeli laboratories constituted a very good way to:

(1) Learn the use, preventive care and maintenance of modern laboratory instrumentation, both in molecular biology and on the use of modern computers, including literature searches through electronic libraries and the Internet. All these capabilities are of special importance in Tashkent, due to their isolation from more active and stimulating academic/research centers.

(2) Interact with a large number of BGU scientists with expertise in fields related to their work. It also facilitated the preparation of new research proposals (CA21-022, currently at the pre-negotiation stage, and CA23-042, decision pending). This project was their first opportunity to plan and carry out a long-range research program in coordination with foreign colleagues. Prior to the project, the Uzbek Institution lacked mechanisms to administer project funds and a number of difficulties had to be overcome, although several of them still remain. Thus, the purchase of equipment from Israel (due to difficulties in Uzbekistan) and the lengthy discussions of scientific and research organization character, contributed markedly to increase the scientific and administrative skills acquired during this project and stand as an excellent foundation for future R&D work at the Uzbek institution.

In practical terms, the impact of this project was to improve the capacity to develop native leguminous species into productive crops in desert areas affected by drought and salinity. The highly stress tolerant strains of N_2-fixing bacteria isolated during this project constitute a valuable collection which could be put to use within a relatively short time in Uzbekistan and other similarly drought affected countries in the world.

G) Project Activities/Outputs

In June 1999 the Israeli PIs visited the Uzebek laboratory and took part in a collecting expedition to the Kyzil-Kum desert.

Drs. Khakimov and Shakirov spent the period between May and October 2000 in Sede Boqer in the laboratories of Drs. Soares (Microbiology) and Lips (Plant Sciences) to characterize at molecular level the bacterial strains isolated in Uzbekistan, and to carry out plant inoculation experiments in the greenhouse.

In 2002, Drs. Khakimov and Shakirov spent the summer period at BGU, to train in the methodology of AO and XDH testing and to carry out experiments that required the use of GC. During this period, they traveled to an International Microbiology Conference in Paris (August) where their results were presented.

Publication of some of the results is in preparation.

H) Project Productivity

The project has achieved the goals that were to be fulfilled during the allocated time span. As described below, the work carried out opened new and lines of work which will continue, both a laboratory and at field level.

I) Future Work

The project will lead to future work, presumably financed by the Global Environmental Fund and other funding agencies. Within a few years, some of the microorganism described in this project will be put to practical use in desert agricultural fields. Field experiments initiated under this program are still in progress and will be concluded in Uzbekistan. Other experiments will be repeated with a higher number of replicates in order to clarify the statistical significance of some of the results obtained and finalize the data for publication. The bacterial isolates will be further studied and used in new plant inoculation studies with the aim of increasing plant productivity in arid areas. Comprehensive field trials with slow-growing desert plants require a number of growing seasons.

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